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Effect of squalene synthase gene disruption on synthesis of polyprenols in *Saccharomyces cerevisiae*

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Abstract Biosynthesis of polyprenols was investigated in a wild-type strain of *Saccharomyces cerevisiae* and a squalene synthase deficient strain auxotrophic for ergosterol. The quantitative data showed that disruption of squalene synthase gene caused a 6-fold increase in the synthesis of polyprenols in vitro in comparison with the wild-type strain. Microsomal preparation from the deleted strain only slightly reacted to the additional exogenous FPP, while that from the wild-type strain presented a 4-fold increase of polyprenol synthesis. Restoration of ergosterol synthesis, by introducing *ERG9* functional allele into the deleted strain resulted in a significant lowering of polyprenol synthesis, indicating the immediate shift of the common substrate (FPP) to the sterol pathway. The role of squalene synthase in the regulation of polyprenol synthesis and 'flow diversion hypothesis' is discussed.

Key words: Squalene synthase; *cis*-Prenyltransferase; Polyprenol; *S. cerevisiae*

1. Introduction

All eukaryotic organisms share the common mevalonic acid pathway to synthesize sterols, dolichols and ubiquinones, as well as isoprenoids covalently linked to proteins, heme *a* and tRNAs [1–4]. The amount of available farnesyl diphosphate (FPP) plays a central role in the mevalonate pathway since FPP is the last common substrate for the synthesis of all the end products. Investigations performed in the last decades have established that the major regulatory enzyme of the initial pathway is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [5–7]. However, exposure of a number of different experimental systems, most notably rapidly differentiating cells or tissues, to inhibitors of HMG-CoA reductase has demonstrated that the synthesis of ubiquinone and dolichol are regulated by a processes distal to HMG-CoA reductase. The most obvious candidates for such a regulatory role are the first committed steps in the biosynthesis of these compounds, i.e. the enzymes utilizing FPP as a substrate.

One of the proposals of branch-point regulation is known as 'flow diversion hypothesis' [8]. In this model regulation is supposed to be mediated by the different affinities of the branch-point enzymes for FPP. The proposed model predicts that squalene synthase should have a low affinity for FPP compared to *cis*- and *trans*-prenyltransferases. This, in turn, would mean that increase and decrease in the pool of FPP would only affect the synthesis of sterols, since the other branch-point enzymes are fully saturated even at low substrate concentration. Furthermore, almost no investigations have been performed that were designated to actually measure whether the available pool of FPP varies under different conditions known to affect the biosynthesis of isoprenoid compounds.

Yeasts synthesize abundant amounts of sterols and *Saccharomyces cerevisiae* is especially suitable for biochemical and genetic studies. It is easy to culture *S. cerevisiae* under controlled respiratory and defined nutrient conditions and to isolate mutants blocked at defined steps in a biosynthetic pathway. For the investigations we used a sterol auxotrophic strain bearing a disrupted squalene synthase gene (*ERG9*) to verify whether the block in sterol branch results in the increase of FPP pool and whether this in turn would influence the activity of *cis*-prenyltransferase.

2. Materials and methods

2.1. Chemicals

Polyprenol and dolichol standards were from the collection of polyprenols of the Institute of Biochemistry and Biophysics (Warsaw). [^{14}C]Isopentenylpyrophosphate (52 mCi/mmol) was from Amersham. All other reagents used were of analytical grade.

2.2. Yeast S. cerevisiae strains

FL 100 MAT a (ATCC 28383) was from F. Lacroute collection, squalene synthase deleted strain was *erg9*::*HIS3 ura3-1,his3-1,leu2-1 ade2-1*, *aux32*. Aerobic growth is dependent on the additional aux32 mutation allowing sterol uptake in aerobiosis [9,10]. The wild-type copy of *ERG9* gene was introduced into the above strain by crossing.

2.3. Media and growth conditions

Yeast cells were grown on YPG medium (1% bacto peptone, 1% yeast extract, 2% glucose). Cells were grown at 28°C with vigorous agitation. Medium for *erg* mutant was supplemented with ergosterol (2 mg/ml) in 1% Tween 80. The yeast microsomal fraction serving as an enzyme source for polyprenol synthesis was prepared as described in [11], the fraction used for all lipidic compound synthesis was obtained after discarding debris after breaking yeast cells with glass beads.

2.4. Synthesis of polyprenols in vitro

The incubation mixture contained, in a final volume of 250 μl, 50 mM sodium phosphate buffer, pH 7.3, 0.5 mM $MgCl_2$, 20 mM β-mercaptoethanol, 10 mM KF, 3×10^5 cpm [^{14}C]IPP and 500 μg of respective protein fraction. FPP (10 μg) was added where indicated. The 90-min incubation at 30°C was terminated by the addition of 4 ml of chloroform/methanol, 3:2. The protein pellet was removed by centrifugation and the supernatant was washed three times with 1/5 volume of 10 mM EDTA in 0.9% NaCl. The organic phase was concentrated under a stream of nitrogen and subjected to thin-layer chromatography and autoradiography [12].

2.5. Chromatography

Thin-layer chromatography was performed on Kieselgel 60 plates (Merck) developed with benzene/ethyl acetate 95:5 (v/v) and HPTLC

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Abbreviations: FPP, farnesyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A

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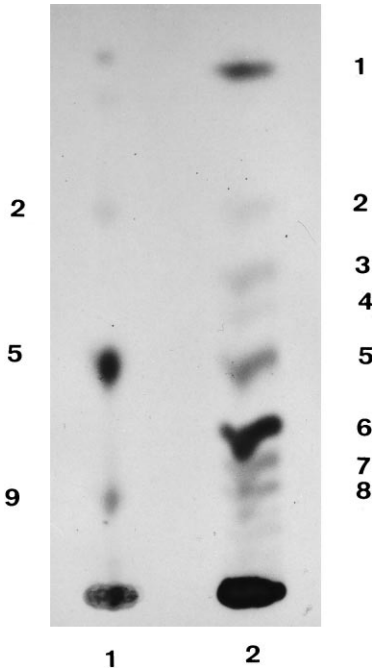

Fig. 1. Autoradiogram of TLC of radioactive lipid compounds synthesized in vitro: *erg9::HIS3* (lane 1) and *FL 100* (lane 2). The lipophilic incubation products were isolated and subjected to chromatography as described in Section 2. Radioactive products were identified relative to unlabeled standards. Synthesized products: 1: squalene; 2: ubiquinone; 3 and 4: quinones; 5: polyprenols of 11–16 isoprene residues; 6: lanosterol; 7: unidentified sterol; 8: ergosterol; 9: farnesol.

RP-18 precoated plates with concentrating zone developed with acetone containing 50 mM H_3PO_4.

3. Results

Microsomal fractions from yeast strain *FL100* and sterol auxotrophic *erg9::HIS3* strain were incubated with [^{14}C]isopentenyl diphosphate. Fig. 1 shows a thin layer autoradiogram of incubation products. As seen in lane 1 (*erg9::HIS3*) there is only one major radioactive product (polyprenols comprising 11–16 isoprene residues [13]) while lane 2 (*FL100*) demonstrates a whole span of radioactive lipid compounds quinones, polyprenols, ergosterol and sterol precursors.

FPP is a common precursor for the synthesis of polyprenols and sterols. We wanted to check whether the block in sterol pathway will influence the level of polyprenol synthesis. The autoradiogram presented in Fig. 2 clearly indicates that disruption in squalene synthase gene resulted in the increase of polyprenol synthesis. Moreover, addition of FPP to the incubation mixtures revealed that the amount of farnesyl diphosphate is the rate limiting factor for the synthesis of polyprenols. The enhanced level of available FPP in the strain deficient in squalene synthetase saturated the *cis*-prenyltransferase which caused the limited responce to the additional amount of FPP. On the other hand, wild-type yeast *FL100* reacted to the exogenous FPP by a considerably elevated synthesis of polyprenols. Quantitative results obtained by scanning of the autoradiogram shown in Fig. 2 are presented in Table 1.

Table 1
Polyprenol in vitro synthesis in wild type and a strain with disruption in squalene synthase gene

FL 100		*erg 9::HIS3*	
FPP		FPP	
–	+	–	+
0.720	2.930	4.356	4.850

Autoradiogram of Fig. 2 was scanned by laser densitometer ultroscan XL. Numbers represent the sum of absorption of individual polyprenols synthesized by wild-type *FL100* and *erg9::HIS3* strains. Incubation and purification of radioactive lipids as in Section 2.

To make sure that the effect of increased synthesis of polyprenols in strain *erg9::HIS3* is strictly connected with the block in sterol pathway caused by the deletion of squalene synthase gene and the flux of common substrate to the polyprenol synthesis we introduced into the above strain the wild copy of the squalene synthase gene. Results presented in Fig. 3 show that the level of polyprenol synthesis in *erg9::HIS3* with the wild copy of the *ERG9* gene dropped to the level of wild-type strain *FL100* (compare Fig. 2, lane 2) and is significantly lower than for *erg9::HIS3*. In addition, we verified that the complemented strain is able to synthesize ergosterol, in contrast to the parental *erg9::HIS3* (data not shown).

4. Discussion

There is gathering evidence that enzymes directly utilizing FPP as a substrate may play the decisive regulatory role. Squalene synthase (SQS) [14] and *cis*-prenyltransferase are the first committed enzymes in the sterol and dolichol biosynthesis. The most commonly accepted proposal for branch-point regulation is known as the 'flow diversion hypothesis' [8,15]. It assumes regulation by the different affinities of the branch-point enzymes for FPP. The model predicts that squalene synthase has a low affinity for FPP compared to *cis*-prenyltransferase. In consequence it would mean that increase and decrease in the pool of FPP would only affect the syn-

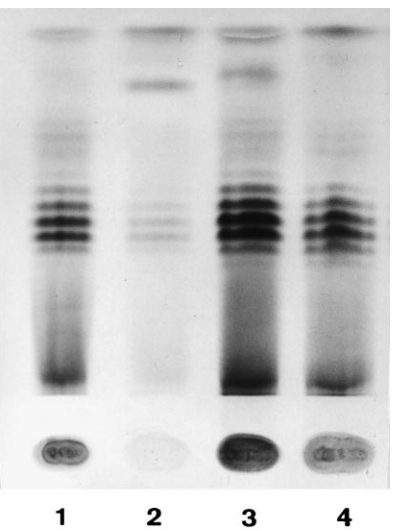

Fig. 2. HPTLC (RP-18) of radioactive polyprenols synthesized in vitro. Strain *FL 100* (lane 1 and 2) and *erg9::HIS3* (lane 3 and 4) with (lane 1, 3) and without (lane 2, 4) exogenous FPP. ^{14}C-labeled polyprenols were detected by autoradiography. Incubation, lipid purification and chromatography as in Section 2.

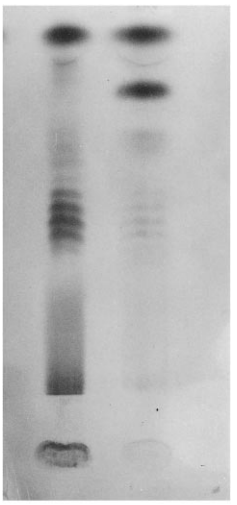

Fig. 3. Autoradiogram of HPTLC of polyprenols synthesized by strain *erg9::HIS3* (lane 1) and the same strain with introduced wild copy of squalene synthase gene (lane 2). Polyprenols were isolated and analyzed as in Section 2.

thesis of sterols, since the other branch-point enzymes are saturated at low substrate concentration.

In our investigations we used two yeast strains, i.e. wild type and a strain with a disrupted squalene synthase gene copy. Results show that the wild-type yeast strain synthesizes a whole span of radioactive lipid compounds while *erg9::HIS3* synthesizes practically only polyprenols. The autoradiogram presented in Fig. 2 clearly indicates the elevated polyprenol synthesis by the strain with squalene synthase gene disruption (lane 4) in comparison with the wild-type strain (lane 2). Quantitative data obtained by scanning of the autoradiogram (Table 1) show a 6-fold higher level of polyprenol biosynthesis. They also show limited (about 11%) increase in polyprenol synthesis in *erg9::HIS3* strain upon addition of the exogenous FPP. FPP synthase (FPPS) is mainly present in cytoplasm. Nevertheless, investigations demonstrate that FPP synthase activity is associated with other subcellular compartments. Runquist et al. and Ericsson et al. [16,17] found that extensively washed rat liver microsomes contained FPP synthase activity and that FPP produced could be used by both squalene synthase and *cis*-prenyltransferase present in the membranes. We presume that FPP amounts present in microsomal fractions prepared from *FL100* and *erg9::HIS3* strains reflect the situation in vivo.

The results show that the hypothesis that *cis*-prenyltransferase is saturated at low FPP concentration [8] and is not influenced by fluctuations in FPP level does not hold. The increase in the amount of farnesyl pyrophosphate in the *erg9::HIS3* strain allowed for the 6-fold higher level of polyprenol synthesis in comparison with the wild-type strain. Furthermore, supplementation of *FL100* microsomal fraction with exogenous FPP and the resulting 4-fold increase in the polyprenol synthesis strongly contradicts the presumption of *cis*-prenyltransferase saturation at low concentration of FPP. The 'flow diversion hypothesis' predicts low squalene synthase and high *cis*-prenyltransferase affinity for FPP. This means that *cis*-prenyltransferase competes more efficiently for the common substrate. Hence, in the wild-type strain *cis*-prenyltransferase should be saturated to a greater extent, which we do not observe.

Moreover, the hypothesis assumes that there exists a common pool of FPP in the cytosol and that this pool is available for all the branch-point enzymes and regulation proceeds by differentiated affinities of the enzymes for FPP. However, it is conceiveable that SQS interacts with FPPS forming a complex. Hence, there could be a direct transfer of FPP from FPPS to SQS (for which FPP is the only substrate) and *cis*-prenyltransferase might have restricted access to FPP. Furthermore, *cis*-prenyltransferase needs two substrates: FPP, only as a primer, and IPP residues for consecutive additions to extend the polyprenol chain, thus interactions of *cis*-prenyltransferase and FPPS may be weaker. As a result *cis*-prenyltransferase is not saturated with FPP. Therefore, depending on the regulation of SQS (synthesis/degradation), less or more produced FPP could be available for *cis*-prenyltransferase, thus affecting polyprenol synthesis. Indeed, the *ERG9*-encoded protein contains a predicted PEST (proline, glutamic acid, serine, threonine) consensus sequence present in many proteins that have a short half-life [18].

Taken together, these results strongly suggest that squalene synthase activity could be the key step in directing the FPP flux either to sterol or polyprenol pathways.

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